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Regional Incorporation and Site-Specific Differentiation of Striatal Precursors Transplanted to the Embryonic Forebrain Ventricle

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Summary

The developmental potential of neural progenitors derived from the E13.5–E14 lateral or medial ganglionic eminences (LGE and MGE, respectively) or the E12 ventral mesencephalon (VM) was examined in a cross-species transplantation model. After injection into the E15 rat forebrain ventricle, mouse LGE progenitors (unlike those of the MGE or VM) were consistently integrated into the host striatum, expressing neurochemical phenotypes and axonal projections characteristic of striatal projection neurons. Additionally, both LGE and MGE precursors displayed widespread incorporation into distinct forebrain and midbrain structures, whereas the more caudally derived VM cells were largely confined to midbrain structures. These results suggest that many LGE precursors are positionally specified for striatal incorporation, while a portion also possess greater potential reflected in more widespread integration following intraventricular injection.

Introduction

The mammalian striatum is the principal component of the basal ganglia, a group of subcortical nuclei involved in the processing of cortical information relating to both complex thought and movement (Alexander and Crutcher, 1990; Gerfen, 1992). The neurons that make up this major forebrain structure arise from the ventral telencephalon and become postmitotic between embryonic day 13 (E13) and E22 in the rat (Bayer, 1984). Previous investigators have suggested that neural precursors located within both the medial and lateral ganglionic eminences (MGE and LGE, respectively) contribute to the growth and development of the striatum (Smart, 1976; Smart and Sturrock, 1979; Lammers et al., 1980; Fentress et al., 1981). Indeed, cell suspension transplants containing both the MGE and LGE from E14–E15 embryos have been shown to develop striatal characteristics after grafting into the adult rat striatum (Wictorin, 1992). Within such transplants, the striatum-like areas express many, if not all, of the neuronal phenotypes typical of the normal striatum (Campbell et al., 1995). However, these regions occupy only about one-third of the graft's total volume, with the other two-thirds containing neurons originating from nearby telencephalic structures such as the cortex and globus pallidus. Recent transplantation studies have indicated that grafts derived from the selective dissection of the E14–E15 LGE give rise to structures that are highly enriched in striatum-like tissue after implantation into the adult rat brain, while MGE grafts of a similar age are consistently devoid of striatal phenotypes (Pakzaban et al., 1993; Deacon et al., 1994a; Olsson et al., 1995). Unlike MGE grafts, LGE transplants are characterized by broad regions (up to 80% of the graft volume) expressing neurochemical markers typical of the striatum, as well as by axons sent out to innervate the host globus pallidus (Deacon et al., 1994a; Olsson et al., 1995). The final differentiated state of the grafted LGE cells does not appear to depend fully on placement into the host striatal environment, since grafts located in heterotopic locations such as the cerebral cortex or midbrain were also observed to differentiate extensively into striatal phenotypes (Olsson et al., 1995). These results suggest that, at E14–E15, neural precursors within the LGE are largely specified to give rise to striatal neurons, many of which will eventually bear the characteristic projection neuron phenotype. In vitro studies using dissociated cultures of LGE and MGE precursors (cultured either together or separately) also support the notion that at least a portion of the neural progenitors within the E14–E15 LGE are specified for a striatal phenotype, in this case 32 kDa dopamine- and cAMP-related phosphoprotein (DARPP-32) expression (Ehrlich et al., 1990; Nakao et al., 1994).

Neural precursors within the ganglionic eminences may, however, possess a wider developmental potential than has been demonstrated by the transplantation experiments in adults. In this respect, immature and, in particular, embryonic CNS environments that are actively undergoing neurogenesis, migration, and differentiation may reveal the potential of striatal progenitors within a developmentally more relevant context. We have developed an in utero cross-species transplantation approach in order to determine the influence of the embryonic environment on the regional integration and subsequent differentiation of neurochemical phenotypes and axonal projection patterns of E13.5–E14 mouse (~E15–E15.5 in the rat) striatal progenitors. This grafting paradigm uses dissociated E12–E14 mouse LGE, MGE, or ventral mesencephalic (VM) progenitors (as detected by the mouse-specific neural antibodies M6 and M2) and introduces them into the forebrain ventricle of rat embryos at a similar developmental stage (E15–E15.5). We show here that mouse progenitors injected into the forebrain ventricle are capable of incorporating into the brain parenchyma and of forming regionally restricted cellular chimeras in the recipient rat brain. The results indicate that precursors derived from the LGE (unlike those from the MGE or VM) consistently incorporated into the developing host striatum and appeared to differentiate into striatal neurons, which suggests that at least a portion of the LGE cells are positionally specified for stria-

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tal incorporation after transplantation in utero. However, LGE progenitors were also seen to integrate into distinct nonstriatal forebrain and midbrain regions and subsequently undergo a pathway of differentiation appropriate to the nucleus in which they resided, suggesting that local environments within the embryonic CNS may influence the final differentiated state of transplanted neural precursors.

Results

Regional Integration of M6-Positive Mouse Neural Cells

Dissociated mouse neural precursors derived from the E13.5–E14 LGE and MGE or E12 VM were injected into the E15–E15.5 rat forebrain ventricle, and the brains were analyzed 1–5 weeks later (at postnatal day 0 [P0]–P28) using the mouse-specific neural markers M6 and M2 to trace the transplanted cells. Neural precursors derived from the mouse ganglionic eminences and VM were found to be extensively integrated into host rat brain nuclei (Figures 1–3), displaying regionally restricted patterns of incorporation following injection into the forebrain ventricle. Interestingly, the incorporated mouse cells often appeared to respect the borders of the nucleus in which they resided, showing little or no mixing with adjacent nuclei (see Figures 3A and 3B). In addition to the specific integration of mouse cells within the host brain parenchyma, clumps or balls of M6-stained tissue were frequently found attached to the ventricular walls; these clumps were often observed to be accompanied by streams of M6-positive cells incorporated into the parenchyma (Figures 1E and 1F).

The most consistent site of M6-positive cellular incorporation of LGE progenitors was into the striatal complex (16 out of 17 animals; Figure 1A; Table 1), where the extent of integration varied between animals from extensive to only a few scattered clusters (see Figures 1 and 2). M6-positive cells are outlined by the reaction product, while the cytoplasm is unstained, giving the impression of holes in the immunopositive tissue, as the M6 antibody stains only the cell membrane (see Figure 1B). The M6-stained cellular profiles within the striatum were typically 18–20 μm in diameter and appeared in clusters surrounded by a dense neuropil staining that was often observed to ensheath the host myelinated fiber bundles coursing through the striatum (see Figure 1B). In addition, M6-positive processes (presumably axons) were also observed to enter adjacent myelin bundles. Injections of [^{3}H]thymidine given to the mothers bearing LGE-transplanted embryos on E18 (i.e., 3 days after transplanting LGE cells) resulted in the labeling of M6-positive cellular profiles located within the striatum (see Figure 1C), suggesting that some of the injected mouse LGE cells that integrated into the striatum continued dividing for at least 3 days after transplantation.

Although LGE cells were consistently found to be incorporated into the striatum, many of these precursors were also found in distinct forebrain and midbrain regions. Other telencephalic sites of M6-positive incorporation included the septal complex (extensive in certain animals; Figures 1D and 2) and the cerebral cortex (Figures 1E and 2) (see Table 1). In the diencephalon, several nuclei localized close to the third ventricle exhibited very specific incorporation of LGE cells. For example, the parafascicular nucleus (Figure 3B) and particularly the periventricular and mamillary nuclei of the hypothalamus (Figure 3C) were frequently found to have M6-positive cellular integration (Table 1). Furthermore, periventricular midbrain structures were also observed to contain LGE cells (Table 1). The patterns of cellular incorporation did not change noticeably between P0 and P28 (Table 1), which suggests that they were largely established during the 1 week period between transplantation at E15 and birth.

In contrast to the LGE cells, MGE precursors rarely integrated into the striatum. Thus, only 3 out of 10 animals showed any detectable M6-positive cellular integration into the striatal complex (Table 2). The septum, basal forebrain, and hypothalamus appeared to be the most common sites for mouse MGE cell integration (Table 2). Again, distinct periventricular thalamic nuclei possessed M6-positive profiles, including the intralaminar centrolateral and paracentral nuclei (see Figure 3A). Analysis of midbrain incorporation by MGE cells was precluded by the fact that the present material did not include brain regions caudal to the diencephalon. Thus, the most clear-cut difference in the patterns of incorporation between LGE and MGE precursors was that the LGE cells much more consistently gave rise to M6-positive cellular integration within the striatum, while both cell types incorporated into broad regions of the developing brain (compare Tables 1 and 2).

In contrast to the widespread incorporation observed for LGE and MGE progenitors, the integration of precursors derived from the E12 VM (roughly at a similar developmental stage as the E13.5–E14 LGE or MGE cells) was largely confined to nontelencephalic regions. Thus, only 2 out of 6 animals exhibited any integration into the telencephalon, with cells located in the cortex and septum (but not the striatum), while the vast majority of these animals displayed incorporation in the hypothalamus and midbrain (Table 2). These midbrain precursors were observed to form balls in the ventricles as far back as the fourth ventricle. However, when these cellular aggregates occurred in the lateral ventricles, they were not accompanied by streams of incorporating cells, as was seen with LGE and MGE cells (e.g., see Figures 1E and 1F). These findings suggest that neural precursors taken from different anterior–posterior regions of the developing CNS do not possess equal capacity for incorporation into particular subregions of the ventricular wall.

Specific Outgrowth of M6-Positive Fibers from Integrated Mouse LGE Cells

LGE cells incorporated into the host striatum exhibited axonal projection patterns characteristic of striatal projection neurons, with fibers terminating in both the pallidal complex (including the globus pallidus and entopeduncular nucleus; 7 animals) and the substantia nigra (5 animals) (see Figures 2 and 4A–4D). This fiber staining was most evident in the animals analyzed at P14 or older; however, weak fiber staining was also detected in animals analyzed

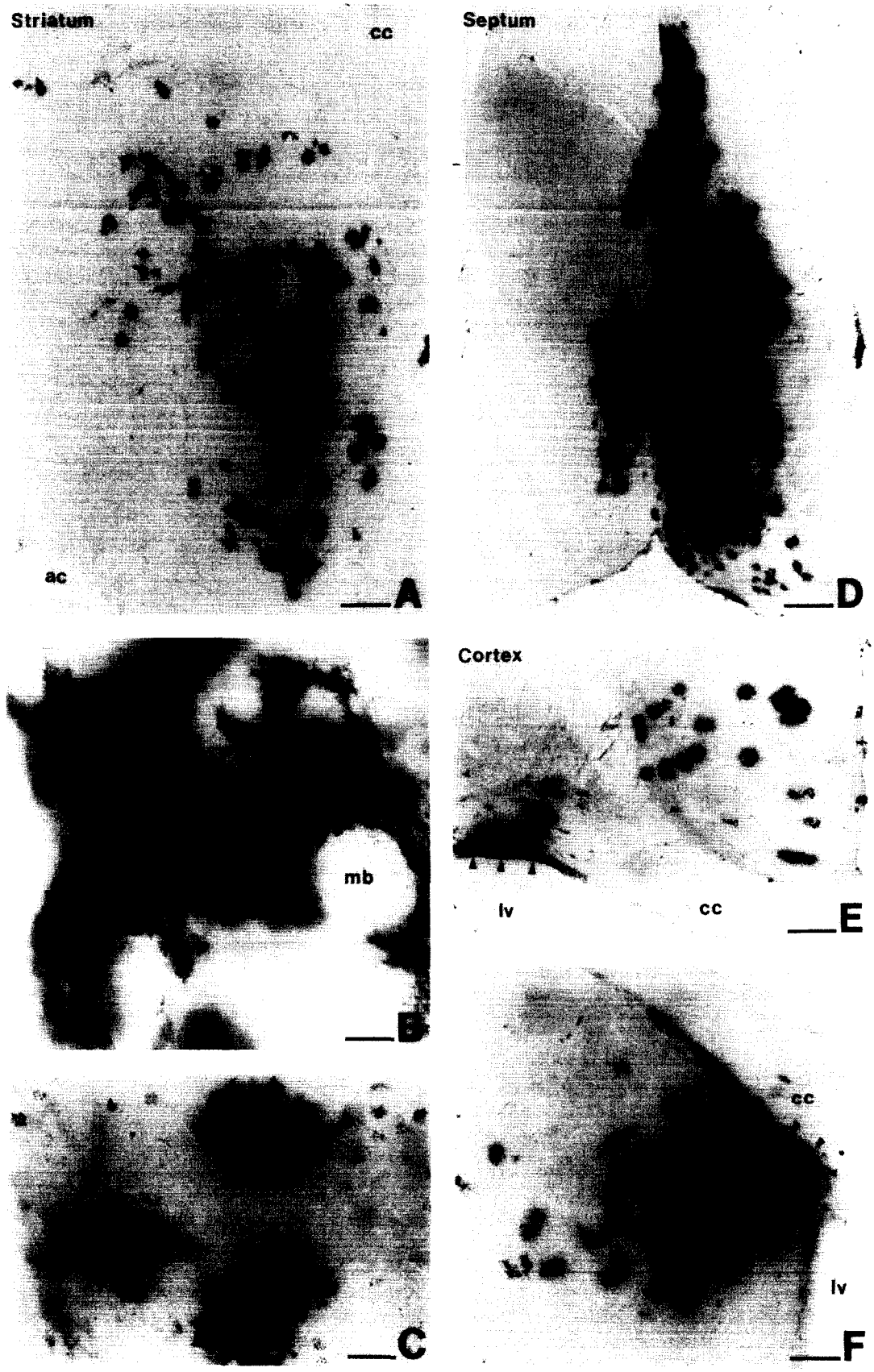

Figure 1. Integration of M6-Positive Cellular Profiles in Telencephalic Nuclei after Injection of Mouse LGE Precursors into the Forebrain Ventricle of E15 Rat Embryos

(A) Low power view of M6 staining in a coronal section of the striatum from an animal sacrificed at P28.

(B) Higher power view of M6-positive cellular profiles incorporated into the striatal gray matter and sending processes around myelinated fiber bundles (mb) passing through the striatum (processed at P28). Note the staining pattern of M6 at the cellular level, with cellular processes and the cell body membrane stained and the cytoplasm unstained (giving the impression of holes in the immunopositive tissue).

(C) M6-positive cellular profiles in the striatum (at P23) containing silver grains (arrowheads) after an injection of [^{3}H]thymidine into the pregnant mother at E18 (3 days after transplantation). Note the presence of silver grain clusters in the surrounding nonstained host striatum.

(D) Extensive M6-positive staining in the septum, most notably in the medial septum and the vertical limb of the diagonal band at P28.

(E and F) M6-positive clusters (or balls) of cells attached to the ventricular wall (arrowheads), with streams of M6-positive cells integrated into the cingulate cortex (E) or into the striatum (F) (at P28).

ac, anterior commissure; cc, corpus callosum; lv, lateral ventricle. Bars, 320 μm (A), 25 μm (B), 50 μm (C), 320 μm (D), 225 μm (E), 160 μm (F).

Table 1. Distribution of M6-Positive Cellular Profiles Integrated into the Developing Rat Brain after Injection of E13.5–E14 Mouse LGE Precursors into the Forebrain Ventricle of E15 Rat Embryos

Mother# Rat# (age)	Cortex	Hippocampus	Corpus Striatum	Septum/Basal Forebrain	Thalamus	Hypothalamus	Midbrain
I M4R1 (P7)	–	++	++++ (Stm, Acc)	± (MS)	++ (CM, Pf, VL)	++	+ (CG)
M4R5 (P7)	++	–	+++ (Stm)	–	+ (LD, ZI)	+	ND
M5R1 (P28)	–	+	++++ (Stm, Acc, GP)	–	+ (AntP, VL)	++	++ (CG, RN, SC)
M5R3 (P28)	±	–	–	+ (LS, HDB)	–	+	ND
M5R5 (P28)	+	+	+ (Stm, Acc)	+++ (MS, LS)	+ (AM, Pf)	+	+ (CG, SC)
M5R7 (P28)	+	±	+++ (Stm, Acc, GP)	++++ (MS)	± (AM)	++	± (CG, SC)
M5R9 (P28)	++	–	++++ (Stm, Acc)	+++ (MS, Fim, HDB)	+ (Pf, Ret)	+	+ (CG, RN)
II M6R1 (PO)	–	–	+++ (Stm)	+ (MS)	ND	ND	ND
M6R2 (PO)	–	–	++ (Stm)	+++ (MS)	ND	ND	ND
III M14R1 (PO)	–	–	++ (Stm)	–	ND	ND	ND
M13R1 (P23)	±	–	++ (Stm, GP)	++ (MS)	–	++	± (CG, RN)
M13R5 (P23)	–	±	+ (Stm)	+ (MS)	+ (AD, Pf)	+	+ (CG, RN)
M13R6 (P23)	+	–	+++ (Stm)	+++ (LS, MS)	–	+	± (CG)
M13R7 (P23)	+	+	++ (Acc, OT)	+++ (LS, MS, Fim, HDB)	+ (Pf)	++	+ (CG)
IV M15R1 (P14)	++	–	++ (Stm)	+ (MS)	–	+	–
M15R2 (P14)	–	–	+++ (Stm)	–	–	–	–
M15R3 (P14)	+	±	+ (Stm)	–	–	±	–
Median score	±	–	++	+	+	+	+

Qualitative analysis of M6-positive cellular incorporation after transplantation of LGE cells in utero. Roman numerals in the left column refer to the grafting session. The midbrain analysis in these sections was confined to the rostral half of this region, including the superior colliculus but not as far caudal as the level of the inferior colliculus. ++++, extensive incorporation (i.e., up to or more than half of all the integrated M6-positive mouse cells incorporated into this region and cellular staining covering around one-fifth or one-fourth of the cross-sectional area in a number of coronal sections through the nucleus); +++, rich incorporation; ++, moderate incorporation (i.e., up to one-fourth of all the integrated M6-positive mouse cells incorporated into this region and the cellular staining covering between one-twentieth and one-tenth of the cross-sectional area in at least two coronal sections through the nucleus); +, scattered clusters of M6-positive cells; –, no detectable staining; ND, not determined.

Acc, nucleus accumbens; AD, anterior dorsal thalamic nucleus; AM, anterior medial thalamic nucleus; AntP, anterior pretectal nucleus; CG, central grey; CM, centromedian thalamic nucleus; Fim, fimbrial nucleus of the septum; GP, globus pallidus; HDB, horizontal limb of the diagonal band of Broca; LD, lateral dorsal thalamic nucleus; LS, lateral septum; MS, medial septum-vertical limb of the idagonal band; OT, olfactory tubercle; Pf, parafascicular thalamic nucleus; Ret, thalamic reticular nucleus; RN, Raphe nucleus; SC, superior colliculus; Stm, striatum; VL, ventrolateral thalalmic nucleus; ZI, zona incerta.

at P0 and P7. The extent of M6-positive fiber and/or terminal labeling within the pallidum or substantia nigra was clearly dependent on the amount of M6-positive striatal integration, such that the largest striatal incorporation gave rise to the most extensive axonal outgrowth. M6-positive fibers within the pallidum were often fasciculated, forming bundles of fibers that appeared to arborize into delicate and more diffusely stained terminal networks within the neuropil of the globus pallidus and entopeduncular nucleus (Figures 4A and 4B) as well as the substantia nigra (Figures 4C and 4D). In a number of animals with septal integration, M6-positive fibers could be seen entering and traversing the fimbria-fornix, the normal pathway of septo-hippocampal projections (Figure 4E). However, little fiber staining was detected within the hippocampus itself.

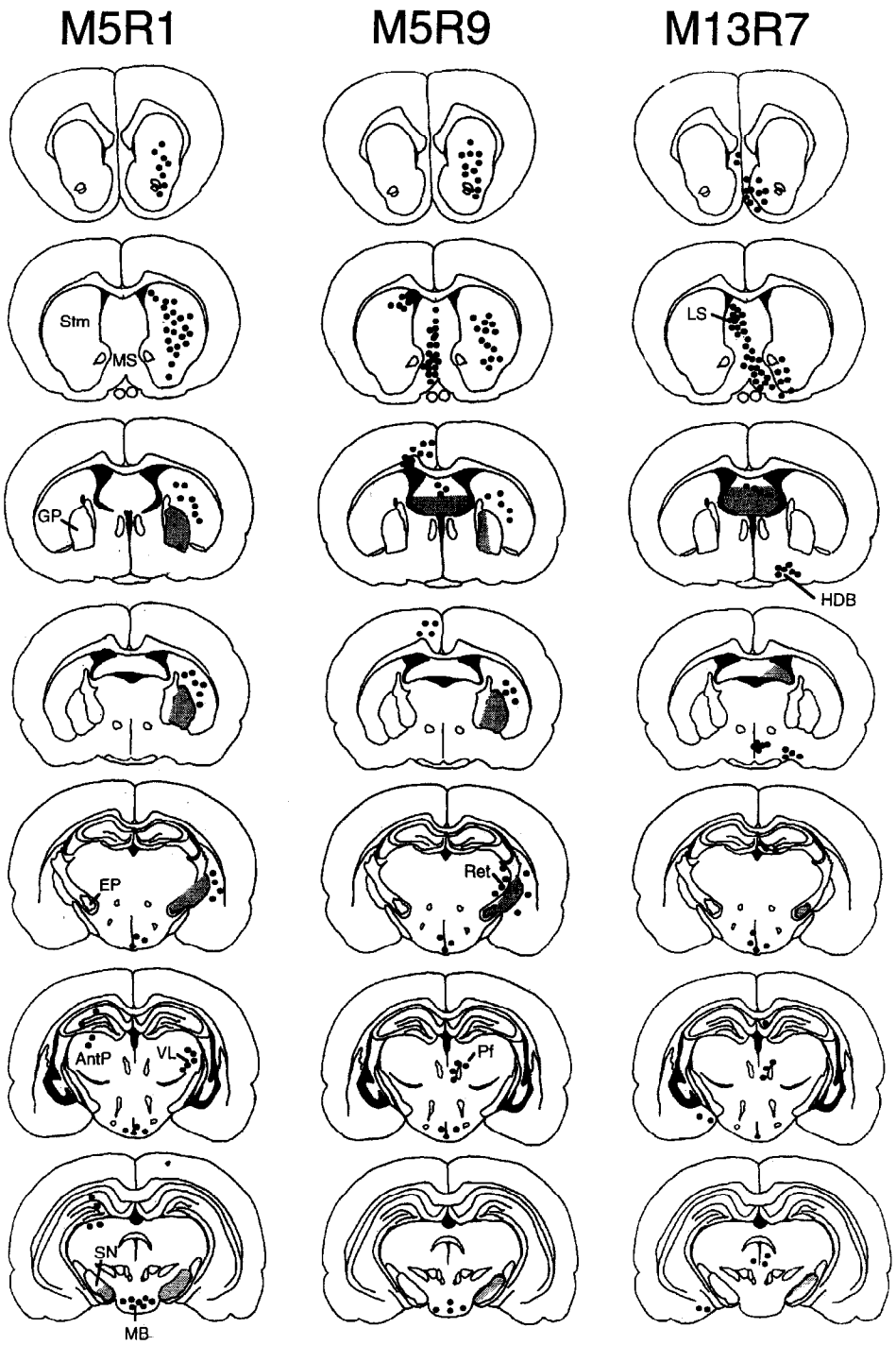

Figure 2. Schematic Representation of M6-Positive Cellular Incorporation of LGE Precursors in Three Representative Recipient Rat Brains

The dots represent clusters or islands of M6-positive cells (not single cells); the stippling denotes areas containing M6-positive fiber staining. Dots do not represent absolute numbers but rather the relative proportions of mouse cells incorporated in different brain areas. Animal M5R1 (sacrificed at P28) exhibited primarily striatal incorporation, while animal M13R7 (sacrificed at P23) had the most extensive incorporation into the septum and basal forebrain nuclei. Animal M5R9 (sacrificed at P28) displayed abundant incorporation in both the striatum and septum (see Tables 1 and 2 for more details). AntP, anterior pretectal nucleus; EP, entopeduncular nucleus; GP, globus pallidus; HDB, horizontal limb of the diagonal band of Broca; LS, lateral septum; MB, mammillary body; MS, medial septum-vertical limb of the diagonal band; Pf, parafascicular nucleus; Ret, thalamic reticular nucleus; SN, substantia nigra; Stm, striatum. Drawings are modified from Paxinos and Watson (1982).

Overlapping Patterns of M2 and M6 Staining

Cells staining positively for the astroglial mouse marker M2 (Lund et al., 1989; Zhou et al., 1990; Zhou and Lund, 1992) were also observed within the recipient rat brain in both the MGE- and LGE-transplanted animals (not investigated in the VM animals). Indeed, as observed in adjacent sections, M6-positive cellular profiles were seen to be extensively colocalized with M2-positive profiles (Figures 5A

Figure 3. Integration of M6-Positive Mouse Cells into Periventricular Diencephalic Nuclei after Injection at E15, as Observed at P28

(A) Integration of MGE precursors into the intralaminar thalamic, centrolateral (CL), and paracentral (PC) nuclei.

(B and C) Integration of LGE precursors into the parafascicular thalamic nucleus (B) and periventricular hypothalamus (C).

3v, third ventricle; fr, fasciculus retroflexus; Hb, habenula. Bars, 450 μm (A), 225 μm (B), 450 μm (C).

and 5B). M2-positive cells were also evident in major white matter tracts that were devoid of M6 staining, such as the corpus callosum. The M2-positive cellular profiles were similar in appearance to those stained with M6 (i.e., membrane staining and a lack of cytoplasmic labeling), with the exception that many of the cell body diameters were only about half the size of the M6-positive profiles, ~8–10 μm (similar to that of astroglia; Figure 5C). In addition, larger, M2-positive profiles (similar in diameter to those observed with M6 staining) were seen in some regions (Figure 5C). In regions where the density of M2-positive integration was lower (e.g., in the globus pallidus), the cells were seen to possess short, ramifying processes with a bushy appearance typical of those seen on astroglia (Zhou et al., 1990; Zhou and Lund, 1992) (Figure 5D).

Interestingly, M2-positive, astroglia-like cells were also observed to overlap, at least in part, with the distribution of M6-positive fiber tracts. Indeed, in all animals stained for both M6 and M2, mouse-derived fiber bundles observed within the pallidum were colocalized with M2-positive profiles (Figure 5D). M2-positive, astroglia-like cells were also seen within the internal capsule, cerebral peduncle, and fimbria in animals exhibiting M6-positive fibers in these axon tracts (Figure 5E). Thus, in certain animals with M6-positive projections to the substantia nigra, M2-positive profiles were detected within the cerebral peduncle as far caudal as the subthalamic nucleus (2 animals) or substantia nigra (1 animal).

Neurochemical Phenotypes of Incorporated LGE Cells

To address whether the LGE cells incorporated into the striatum (which also sent axons to the pallidum and substantia nigra) expressed neurochemical phenotypes characteristic of striatal projection neurons, adjacent sections were double stained with M6 and an antibody directed against DARPP-32 or the calcium-binding protein calbindin (CaBP). DARPP-32 immunoreactivity marks the overwhelming majority of medium-sized striatal projection neurons within the mature striatum (Hemmings and Greengard, 1986; Anderson and Reiner, 1991). In sections double stained for M6 and DARPP-32, many of the M6-positive profiles in the striatum expressed DARPP-32 (Figures 6A and 6B). M6-positive profiles in nonstriatal regions such as the septum or cortex (structures normally very low in DARPP-32 expression) were never observed to be DARPP-32-positive (data not shown), suggesting that only the M6-positive cells that had integrated into the striatum had differentiated neurochemically into striatal projection neurons.

The striatum is characterized by neurochemically and anatomically distinct compartments termed "patch" and "matrix" (see Gerfen, 1992, for review). CaBP is a marker of striatal neurons belonging to the matrix compartment (Gerfen et al., 1985). Sections reacted for M6 and CaBP revealed a number of M6-positive cellular profiles double labeled with anti-CaBP, and the majority of M6-positive cellular profiles appeared to be localized to the CaBP-rich matrix region of the striatum (Figures 6C and 6D). Interestingly, M6-positive cells that occurred in the CaBP-poor patch regions appeared themselves to be CaBP-negative (Figure 6C). Although CaBP is a more widespread marker in the brain than DARPP-32, we did not observe ectopic (or

Table 2. Distribution of M6-Positive Cellular Profiles Integrated into the Developing Rat Brain after Injection of E13.5–E14 Mouse MGE or E12 VM Precursors into the Forebrain Ventricle of E15 Rat Embryos

Mother# Rat# (age)	Cortex	Hippocampus	Corpus Striatum	Septum/Basal Forebrain	Thalamus	Hypothalamus	Midbrain
Medial ganglionic eminence							
I M1R1 (P0)	–	–	–	++ (MS)	–	+	ND
M1R3 (PO)	++	–	± (Stm)	++ (MS)	–	–	ND
M1R4 (PO)	–	–	–	+++ (MS, Fim)	–	++	ND
II M2R2 (P7)	++	–	+++ (Stm, Acc, OT)	+++ (HDB, VP, SI)	–	–	ND
M2R3 (P7)	+++	–	+++ (Stm, Acc)	+++ (MS, LS, HDB, SI)	–	+	ND
M2R4 (P7)	–	–	–	–	++ (LGN,ZI)	±	ND
M3R1 (P28)	–	–	–	–	++ (VM, ZI)	+	ND
M3R3 (P28)	+	++	–	++ (LS)	++ (CL, PC)	+	ND
III M10R1 (PO)	–	–	–	++ (HDB, VP)	–	+	ND
IV M18R2 (P14)	–	–	–	–	++ (AM)	–	ND
Median score	–	–	–	++	–	+	ND
Ventral mesencephalon							
V M19R2 (P28)	+	–	–	+++ (MS, Fim)	++ (AV, LD, Pf)	++	++++ (CG, SN, IC, MRF)
VI M1R2 (P28)	–	–	–	–	–	+	++++ (CG, IC, MRF)
M1R3 (P28)	–	–	–	–	–	+	++ (CG, IC)
M2R6 (P28)	±	–	–	+ (MS)	–	++	+++ (CG, MRF, IC)
M2R9 (P28)	–	–	–	–	–	–	+ (CG)
M2R12 (P28)	–	–	–	–	–	±	++ (CG, IC)
Median score	–	–	–	–	–	+	+++

Qualitative analysis of M6-positive cellular incorporation after transplantation of MGE or VM cells. The extent of cellular incorporation was determined as described for Table 1.

Acc, nucleus accumbens; AM, anterior medial thalamic nucleus; AV, anterior ventral thalamic nucleus; CG, central grey; CL, centrolateral thalamic nucleus; Fim, fimbrial nucleus of the septum; HDB, horizontal limb of the diagonal band of Broca; IC, inferior colliculus; LD, lateral dorsal thalamic nucleus; LGN, lateral geniculate nucleus; LS, lateral septum; MRF, mesencephalic reticular formation; MS, medial septum-vertical limb of the diagonal band; OT, olfactory tubercle; PC, paracentral thalamic nucleus; Pf, Parafascicular nucleus; SI, substantia inominata; SN, substantia nigra; Stm, striatum; VM, ventromedial thalamic nucleus; VP, ventral pallidum; ZI, zona incerta.

inappropriate) expression of this molecule in M6-positive cells located in other brain regions (data not shown).

In many animals, LGE cells were also observed to incorporate extensively into the septal-diagonal band complex. Although the cells localized in the medial septum were not observed to express DARPP-32, some of them, at least, expressed neurochemical markers typical of medial septal neurons, i.e., p75 (the low affinity nerve growth factor [NGF] receptor; Figures 6E and 6G) and the calcium-binding protein parvalbumin (Figures 6F and 6H). The low affinity NGF receptor marks the cholinergic cells present in the septal-diagonal band area (Batchelor et al., 1989), while parvalbumin is present in the GABAergic cells of this nucleus (Kiss et al., 1990). These findings suggest

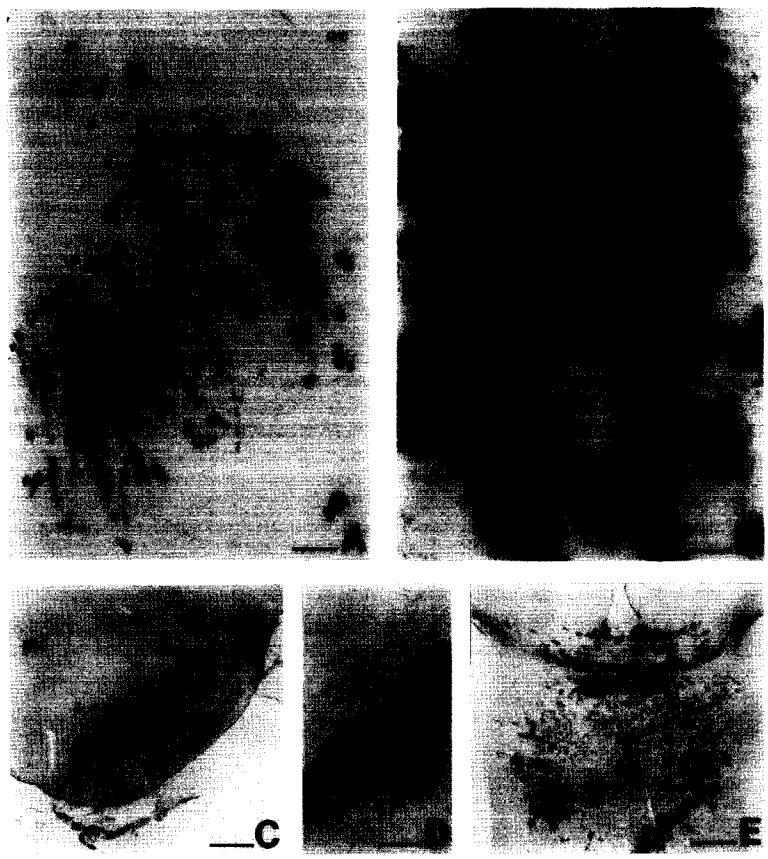

Figure 4. Mouse-Specific Fiber Projections in the Recipient Rat Brain

(A) Coronal section through the striatum at the level of the globus pallidus (GP; border indicated by the dashed line) in an animal that received LGE cells at E15 and was sacrificed at P28. Note the difference in M6 staining between the striatum and GP.

(B) High power view of the boxed area in (A) showing M6-positive fiber bundles as well as thin ramifying processes within the GP.

(C) Fiber staining in the substantia nigra pars reticulata (SNr) in an animal with extensive striatal incorporation of M6-positive mouse LGE precursors.

(D) High power view of the boxed area in (C) showing the particulate staining of M6 in this nucleus (arrowheads point to a thin M6-positive fiber).

(E) M6-positive fibers traveling through the fornix (fx), presumably emanating from the extensive incorporation of LGE precursors into the medial septum in this animal, which was sacrificed at P23 (arrowheads point to M6-positive cells in the caudal septum).

cc, corpus callosum; RN, red nucleus. Bars, 320 μm (A), 38 μm (B), 450 μm (C), 110 μm (D), 450 μm (E).

that, rather than developing into striatal neurons (as was the case in the striatum), at least a portion of the M6-positive LGE precursors that had incorporated into the septal complex differentiated into medial septal neurons. The low affinity NGF receptor, which is normally absent in the striatum, was not detectable in the M6-positive cellular profiles present in the striatum, while single M6-positive striatal cells were observed to express parvalbumin. This latter observation is consistent with the fact that parvalbumin normally occurs in a subpopulation of striatal GABAergic neurons, namely the interneurons (Cowan et al., 1990).

Discussion

The results of the present study demonstrate a remarkable capacity of mouse neural precursors to incorporate into the rat brain after injections into the embryonic forebrain ventricle. Although previous transplantation studies have shown considerable migration of the dissociated and grafted fetal cells within the host brain in neonatal recipients (McConnell, 1985, 1988; Zhou et al., 1990; McConnell and Kaznowski, 1991; Zhou and Lund, 1992), the present study is among the first to demonstrate the capacity of primary neural progenitors to incorporate into the host brain parenchyma and subsequently undergo a site-specific differentiation after intraventricular injections in the mammalian embryo.

Technical Considerations

The interpretation of the present results relies heavily on the ability to detect the transplanted mouse cells within the host rat brain accurately and consistently. The mouse-specific M6 and M2 antibodies used here are highly specific and have been used extensively for this purpose in studies of mouse neural transplants in neonatal or adult rat recipients (e.g., Lund et al., 1985, 1989; Zhou et al., 1990; Wictorin et al., 1991; Zhou and Lund, 1992). Previous studies have demonstrated that the M6 antibody detects an antigen located on neurons both in culture (Lagenaur et al., 1992) and after grafting in vivo (Zhou et al., 1990). With some notable exceptions, such as cerebellar Purkinje cells and spinal motor neurons, most CNS neurons appear to express detectable levels of M6a mRNA (the message encoding for M6), and neurons in telencephalic regions such as the cerebral cortex and striatum are particularly rich in this message (Yan et al., 1993; C. F. Lagenaur, personal communication). Therefore, the distinct patterns of cellular incorporation from either MGE, LGE, or VM cells observed here with the M6 antibody are likely to represent differences in the properties of the constituent neuronal precursors rather than detection of only a subpopulation of transplanted cells expressing M6. Less is known about the M2 antibody, however. An earlier study reported that this antibody recognizes both neurons and glia in culture (Lagenaur and Schachner, 1981). More recent in vivo studies have demonstrated that this antibody

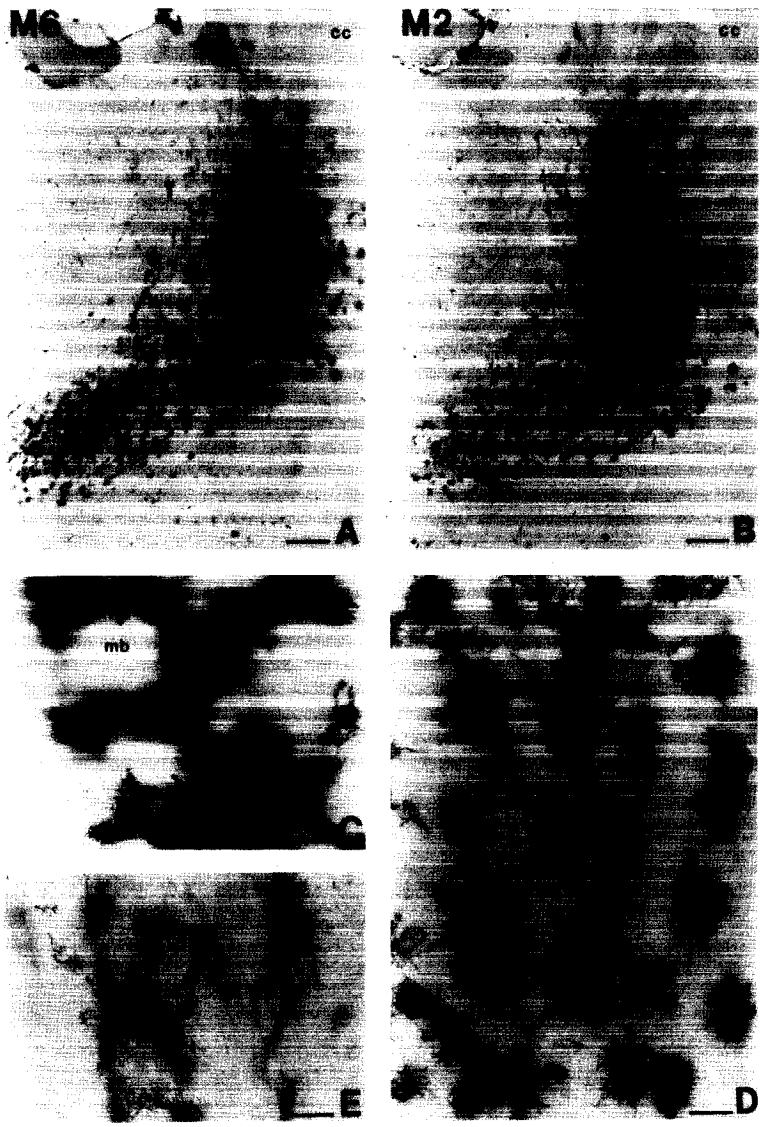

Figure 5. Overlapping Patterns of M6- and M2-Positive Cellular Profiles

(A) M6 staining in a coronal section through the caudal striatum at the level of the globus pallidus (GP) and entopeduncular nucleus (EP) in an animal that received mouse LGE precursors at E15 and was sacrificed at P28. Only a few cellular profiles are present in the striatum, while bundles of fibers are seen to invade the GP and EP, as well as course through the internal capsule (ic).

(B) M2 staining in a section adjacent to that in (A). M2-positive cells are distributed throughout the striatum, GP, and EP.

(C) High power view of M2 staining within the striatum after injection of LGE cells. The M2-positive cells display labeling of their membrane and processes, leaving the cytoplasm unstained; however, many of the cellular profiles are much smaller in diameter (arrowheads). The arrow points to a large cellular profile similar in size to those seen with M6 staining.

(D) M2-positive cells (derived from LGE precursors) in the GP (where M6-positive fibers are evident in an adjacent section) displaying the typical astroglial profile (arrowheads point to cell bodies).

(E) M2-positive cells integrated into the internal capsule (arrowheads point to cell bodies).

cc, corpus callosum; mb, myelin bundle. Bars, 320 μm (A and B), 50 μm (C–E).

recognizes, rather selectively, an antigen present on grafted cells with the appearance both morphologically and neurochemically of astroglia (Lund et al., 1989; Zhou et al., 1990; Zhou and Lund, 1992). The morphological appearance of M2-positive cells in the present study also supports the notion that a considerable portion of these cells represent astroglia.

Host Incorporation of Grafted Mouse Neural Precursors

As mentioned above, extensive and directed migration of intracerebrally grafted neural cells from the site of implantation has previously been observed after transplantation of developing CNS tissue in neonatal and adult recipients (McConnell, 1985, 1988; Sotelo and Alvarado-Mallart, 1986; Zhou et al., 1990; McConnell and Kaznowski, 1991; Zhou and Lund, 1992). In these cases, the donor cells were implanted into the brain parenchyma and observed to undergo rather specific migratory patterns. In the present study, by contrast, the mouse cells were injected into the cerebral ventricle and allowed to flow through the developing ventricles (as evidenced by small clumps of M6-positive cells attached to the walls of the lateral ventricles [bilaterally] and as far back as the third and fourth ventricles) before incorporation into the host brain parenchyma. Similar in utero transplantation paradigms have recently been described in which the donor fetal cells were obtained from E14.5–E16.5 rat embryos and prelabeled with either the mitotic marker bromodeoxyuridine (BrdU; Cattaneo et al., 1994) or the lipophilic dye PKH-26-GL (Fishell, 1995) before injection into the forebrain ventricle. The present results, along with those of Fishell (1995), demonstrate extensive integration and migration of the transplanted forebrain precursors into distinct regions of the developing recipient rat brain after intraventricular injections. The most likely manner in which the donor cells integrate into the host brain parenchyma after introduction into the ventricle is via the ventricular wall. That neural

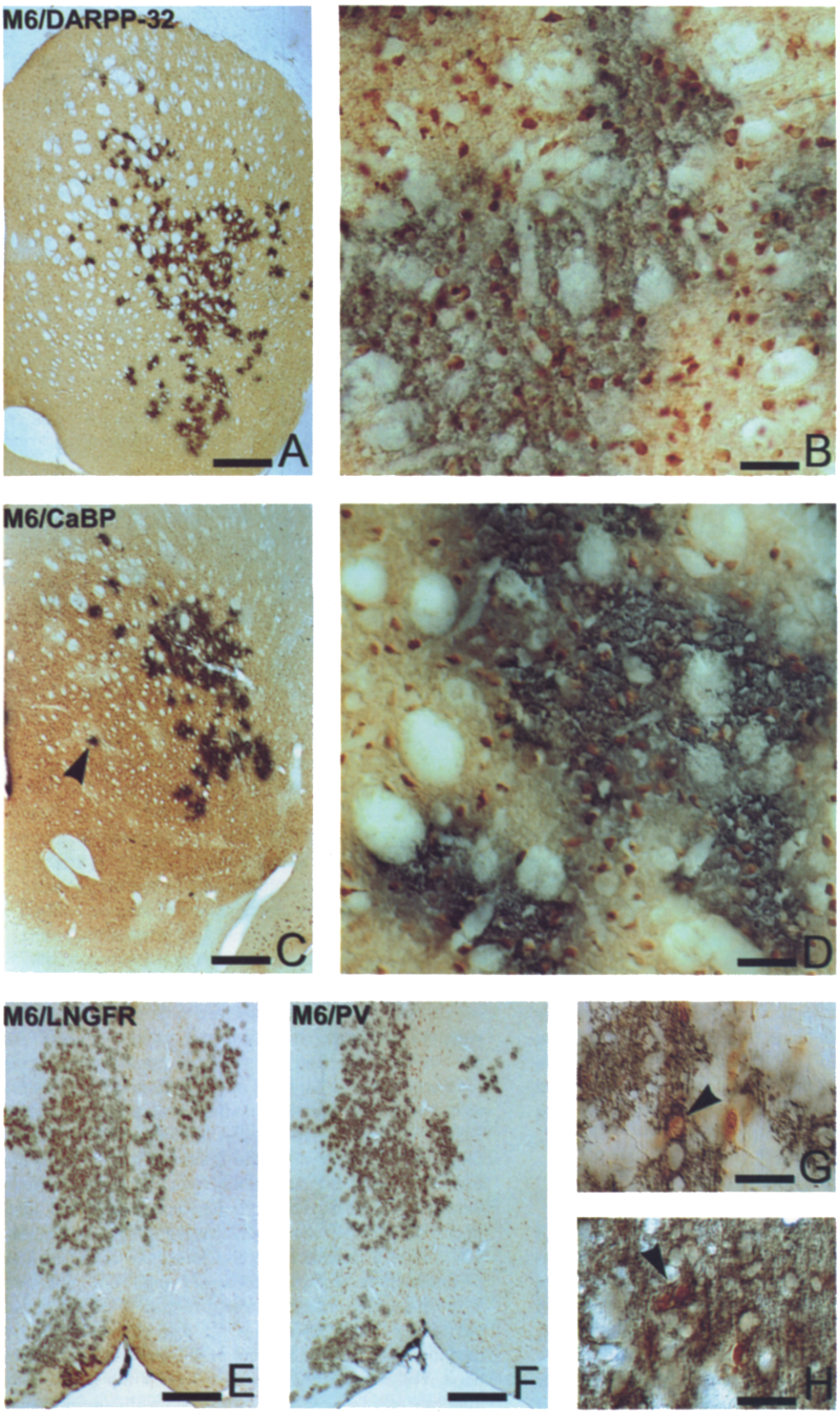

Figure 6. Neurochemical Phenotypes of M6-Positive Cellular Profiles Incorporated into the Telencephalon Following Injection of LGE Precursors at E15

progenitors are capable of incorporating into the ventricular wall has recently been shown by Fishell (1995), where dissociated precursors derived from the E15 mouse LGE or cortical ventricular zone were seen to reincorporate both homotopically and heterotopically into telencephalic germinal zones maintained in vitro in explant cultures. In addition to the extensive incorporation of mouse cells, we also observed balls or clumps of M6-stained tissue attached to the ventricular wall. This observation is similar to that reported by Cattaneo et al. (1994), where precursors labeled prior to transplantation with BrdU formed clumps throughout the ventricular system, with few BrdU-positive cells (i.e., postmitotic around the time of grafting) integrated into the host brain parenchyma. These results suggest that the clumps of M6-stained cells attached to the ventricular walls in our material may in part represent mouse neurons that had become postmitotic prior to, or at the time of, transplantation.

Together, these findings suggest that the specific ability of donor neural precursors to incorporate into the embryonic recipient brain via the ventricular wall requires that they remain in a proliferative state. Accordingly, we observed M6-positive cells in the striatum (which were transplanted at E15) that had incorporated [^{3}H]thymidine after injections given at E18, indicating that some of the cells continued cycling for at least 3 days. Additionally, Fishell (1995) has reported that, in certain cases where cells were incorporated into the ventricular zone in explant cultures, pairs of donor cells could be observed in close proximity to each other. This was possibly the result of a single cell division following the incorporation of a progenitor into the ventricular zone.

Regional Integration of Grafted Mouse Neural Precursors

The E12–E14 mouse neural precursors not only incorporated into the host brain after transplantation in utero but were also observed to populate specifically very distinct brain nuclei. The patterns of incorporation observed with LGE and MGE cells were clearly overlapping, the principal difference being that LGE cells, and not MGE cells, consistently incorporated into the developing host striatum (compare Tables 1 and 2). By contrast, both MGE and LGE precursors incorporated extensively into other forebrain sites, such as the septal complex and several diencephalic and mesencephalic nuclei. Few animals displayed extensive incorporation into dorsal telencephalic structures such as cortex or hippocampus after either LGE or MGE transplants.

Neural progenitors in the ganglionic eminences are clearly not unique in their ability to incorporate with regional specificity into the host brain after injection into the ventricular space, in utero. Progenitors derived from the E12 VM did so also, but they were considerably more efficient at incorporating into the developing hypothalamus and midbrain than into telencephalic and thalamic structures (which was typical of the LGE and MGE cells). This is in spite of the fact that the cells are first introduced into the telencephalic vesicle and must flow caudally in order to incorporate into the brain. These results suggest that populations of neural precursors derived from different anterior–posterior regions of the ventricular wall may exhibit selective affinity for portions of the ventricular wall containing like progenitors, thus resulting in homotopic incorporation.

The present findings are somewhat at variance with those reported by Fishell (1995), where many animals receiving LGE cells exhibited more extensive incorporation into the cerebral cortex than in the striatum. This apparent difference in the extent of cortical incorporation may be due to the age of donors and recipients used in the two studies. In the Fishell (1995) study, the embryos were about 1.5 days older (E16.5) than those used here. Another possibility is that the cells incorporated homotopically into the striatum underwent more extensive proliferation than those positioned heterotopically in the neocortex. This might have diluted out the lipophilic dye in striatal but not cortical cells. As stated by Fishell (1995), this dye is not capable of following the cells for more than three divisions, whereas the mouse markers used here are insensitive to proliferation. In fact, some of the cells that had incorporated into the striatum did continue cell division for at least 3 days after transplantation. It should be pointed out, however, that it is conceivable that the M6 and M2 surface markers used here may have been selectively down-regulated in subpopulations of incorporated mouse cells (e.g., in the cerebral cortex). Thus, with either technique, the lack or relative paucity of cellular incorporation into distinct forebrain nuclei must be interpreted with caution.

Mouse-derived glial cells (as revealed with the M2 antibody) were also seen to incorporate in the developing brain with regional specificity. These cells were observed to overlap extensively with the mouse-derived neurons (as marked by M6 staining), which may provide a hint as to

(A) M6 (blackish-brown reaction product) and DARPP-32 (yellowish-brown reaction product) double staining in the striatum.
(B) High power view of M6/DARPP-32 double staining in the striatum showing a number of M6-positive cellular profiles that also contain DARPP-32 immunohistochemical product.
(C) M6 and CaBP double labeling in the striatum. Note the heterogeneity of CaBP staining in (C), with areas low in CaBP staining (i.e., the patch compartment) surrounded by regions rich in CaBP-postive neurons (i.e., the matrix compartment). While most M6-positive cells were located in the CaBP-rich matrix, cells were also observed in the CaBP-poor patch compartment (arrowhead).
(D) High power view of M6/CaBP double staining taken from the CaBP-rich matrix region. As was the case for DARPP-32, many M6-positive cellular profiles were double labeled with CaBP.
(E and F) M6 staining (blackish-brown reaction product) combined with either the low affinity NGF receptor (E) or parvalbumin (F) (yellowish-brown reaction product) in the septum.
(G) Arrowhead points to an M6-positive cell in the medial septum double labeled for the low affinity NGF receptor.
(H) M6-positive cells in the medial septum double labeled for parvalbumin (arrowhead).
ac, anterior commissure. Bars 530 μm (A and C), 60 μm (B and D), 430 μm (E and F), 55 μm (G and H).

the regional incorporation of these cells. Since radial glia and neurons may be clonally related within the striatal germinal zone (Halliday and Cepko, 1992), it seems possible that the mouse neural precursors may provide their own cellular substrate (i.e., radial glial cells) for the directed migration of mouse neurons observed here. If so, the extensive overlap between M6-positive (putative neuronal) and M2-positive (putative astrocytic) neural elements could be explained, in part, by the fact that radial glia can transform into astrocytes subsequent to the period of neurogenesis (Schmechel and Rakic, 1979; Voigt, 1989).

M2-positive mouse astrocytes were found in many but not all regions where mouse-derived M6-positive fibers were found. It appeared that the further the axons had extended along their trajectory, the fewer the M2-positive astrocytes present. Although these observations strongly suggest that the mouse-derived glial cells may migrate together with the outgrowing axons along their growth trajectory, it is not possible on the basis of the present data to say whether these cells play an active role in the axonal extension or are simply providing support for the growing axons. It should be mentioned, however, that this is not the only transplantation model in which at least a partial colocalization of LGE-derived graft axons and glial elements has been observed. Deacon et al. (1994b) have reported that axons emanating from porcine LGE grafts placed into the adult rat striatum are accompanied by graft-derived glial elements as they extend through the host brain. Additionally, our own results from grafting LGE precursors into the early postnatal striatum have shown that, again, M2-positive glial cells are colocalized with M6-positive graft fibers as far as the host pallidum (M. O. et al., unpublished data).

Site-Specific Differentiation of Incorporated Mouse Neural Precursors

Interestingly, the mouse precursor cells integrated into the host brain parenchyma appeared to undergo a site-specific differentiation. Thus, cells positioned within the host striatum exhibited neurochemical phenotypes and axonal projections characteristic of striatal neurons. In many animals with notable striatal incorporation, M6-positive fibers were found in all principal target nuclei of the striatal projection neurons. Furthermore, many of the M6-positive cellular profiles in the striatum appeared to express both DARPP-32 (a marker of striatal projection neurons; Anderson and Reiner, 1991) and CaBP (found in many striatal neurons, including projection neurons, belonging to the matrix compartment of the adult striatum; Gerfen et al., 1985). The observation that a large number of the incorporated mouse neurons were found in the CaBP-rich matrix is consistent with our finding that at least some of these cells became postmitotic on E18 (within the period over which the majority of striatal neurons leaving the cell cycle contribute to the matrix compartment; van der Kooy and Fishell, 1987). These findings indicate that a considerable portion of the mouse-derived LGE cells that had incorporated into the striatum had differentiated into striatal neurons. By contrast, the mouse LGE precursors that had incorporated into nonstriatal structures such as the septal-diagonal band complex appeared to follow a different developmental pathway. These cells displayed phenotypes typical of medial septal neurons, such as the low affinity NGF receptor or parvalbumin. In the medial septum, these two markers distinguish between the cholinergic and GABAergic subpopulations of septo-hippocampal neurons, respectively (Batchelor et al., 1989; Kiss et al., 1990). Consistent with this, most animals with extensive incorporation into the medial septal nucleus displayed M6-positive fiber bundles coursing through the fimbria-fornix (a major projection pathway for septo-hippocampal neurons), although little M6-positive terminal staining was observed within the hippocampus itself. These findings indicate that the LGE cells that had incorporated into the septal-diagonal band complex appeared to follow a pathway of differentiation akin to their host septal neighbors. In a similar vein, Fishell (1995) found that transplanted LGE cells that had incorporated into the developing cerebral cortex possessed axonal connections with contralateral cortical areas (and to a lesser extent the spinal cord) and that these cells displayed neuronal morphologies typical of cerebral cortical neurons. Thus, local environments in the embryonic CNS may be capable of influencing the final differentiated state of transplanted neural precursors. These results indicate that at least a portion of the precursors within the LGE possess a greater developmental potential than is revealed by the adult transplantation paradigm.

Specification of Neural Precursors in the Ganglionic Eminences

Recent studies analyzing patterns of genes expressed during embryonic development indicate that the telencephalon is regionalized at midgestation stages (e.g., E12.5) with respect to distinct dorsal and ventral domains such as the cortical and striatal germinal zones. Examples of this are the restricted patterns of expression for homeobox-containing genes such as *Emx1* (Simeone et al., 1992), which is restricted to the cortical ventricular zone, and members of the *Dlx* gene family, which are found confined to the ganglionic eminences (Price et al., 1991; Bulfone et al., 1993). Even within the ganglionic eminences, there are regional differences in gene expression, with the homeobox-containing genes *Nkx-2.1* and *Gbx-2* confined exclusively to the MGE (Price et al., 1992; Bulfone et al., 1993). This regionalization is also apparent in the migration patterns of forebrain precursors. In fact, Fishell et al. (1993) have shown that cortical and striatal progenitors in the E15 mouse telencephalon move extensively within their respective germinal zones, but are restricted from crossing the border between them. Transplantation studies using solid grafts derived from selected portions of the developing cerebral cortex further support an early regionalization of the forebrain, and in addition suggest that specific cortical regions (e.g., somatosensory and limbic corticies) are specified even before the influx of afferent inputs (Barbe and Levitt, 1991; Cohen-Tannoudji et al., 1994). These results from solid tissue

transplants demonstrate that the regionalizing cues present within the tissue graft environment are maintained through the transplantation procedure; however, this approach is not capable of challenging single precursors to assess their full developmental potential. One way to address this question is to transplant dissociated progenitors into a potentially novel environment, and thus directly challenge the prior specification of the isolated cell. In this respect, dissociated E14–E15 LGE cells grafted heterochronically into the adult rat striatum appear to differentiate extensively into striatal neurons (Pakzaban et al., 1993; Deacon et al., 1994a; Olsson et al., 1995). The extensive differentiation into striatal phenotypes by LGE cells does not seem to be dependent on a striatal placement, however, since grafts located in the cerebral cortex or midbrain develop in a very similar manner (Olsson et al., 1995). These findings suggest that, when presented with the adult brain environment, whether homo- or heterotopically, dissociated E14–E15 LGE cells maintain their prior striatal specification.

The introduction of dissociated neural precursors into the embryonic ventricular system represents a unique way of assessing the developmental potential of these progenitors with respect to their capacity to incorporate into the developing brain and subsequently differentiate into neuronal phenotypes. The consistent incorporation of mouse LGE cells within the host rat striatum (not seen with either MGE or VM progenitors) after injection into the forebrain ventricle most likely reflects the fact that at least a portion of these precursors are specified prior to transplantation with respect to positional identity, and that this is manifest by homotopic (i.e., striatal) incorporation. This positional specification may be mediated by cell surface molecules, which would allow the LGE cells to associate selectively with like progenitors in the striatal germinal zone of the recipient rat brain. Such a scheme would require that different regions of the embryonic ventricular wall express unique surface molecules, and thus allow for the sorting of donor cells present in the ventricular fluid. In support of this notion, Götz et al. (1994, Soc. Neurosci., abstract) have shown that progenitors derived from subregions of the E14 telencephalic ventricular zone display selective adhesion with each other in reaggregate cultures. Specifically, they found that cortical precursors had a higher affinity for association with each other than with ganglionic eminence precursors, and vice versa. It is likely, however, that not all of the E13.5–E14 LGE progenitors are specified at the time of transplantation and that placement into the embryonic environment reveals this. Thus, unlike the situation in the intraparenchymally placed adult grafts, where specified and unspecified cells would be forced to associate with each other, in the embryonic transplantation model, cells lacking instruction as to their positional identity would be free to sample from the ventricular wall and incorporate into nonstriatal primordia. Once these cells have incorporated, whether into the striatal, septal, or cortical germinal zone, they would likely receive and be receptive to the same signals for migration and differentiation as their host counterparts. The positional specification of these neural precursors may be dependent on their position in the cell cycle, as has been suggested for laminar specification of cortical progenitors (McConnell and Kaznowski, 1991). Cortical precursors late in the cell cycle retain their prior specification, while those in S phase appear to be capable of respecifying in response to local cues. Thus, the patterns of incorporation, including both homotopic (i.e., striatal) and heterotopic cellular integration, for the LGE precursors observed here might be explained, at least in part, by the presence of subpopulations of transplanted progenitors in different stages of the cell cycle. In accordance with this, Acklin and van der Kooy (1993) have recently shown that progenitors in the E17–E19 striatal germinal zone are heterogeneous with respect to their cell cycle kinetics.

Conclusions

The present results demonstrate that a portion of the E13.5–E14 LGE precursors, unlike those taken from the MGE or VM, are positionally specified for striatal incorporation after introduction into the forebrain ventricle. Additionally, both LGE and MGE precursors are capable of a more widespread incorporation, including telencephalic, diencephalic, and mesencephalic regions of the developing brain and subsequent site-specific differentiation, suggesting that ganglionic eminence precursors possess a greater developmental potential than is revealed by the adult transplantation paradigm. This cross-species transplantation model provides a powerful tool for the further study of the developmental potential of neural precursors derived from selected subregions of the developing CNS. Moreover, this technique should make it possible to generate experimentally interesting brain-specific mouse–rat cellular chimeras of progenitor cells obtained from transgenic donors carrying modified or inactivated genes.

Experimental Procedures

In Utero Transplantation

Donor cells for these experiments were obtained from E12–E14 mouse embryos of timed pregnant CD1 mice (BK Universal, Stockholm, Sweden). After freeing the brains of the surrounding mesenchymal tissue, the floor of the telencephalon was exposed, and the dorsal-most aspects (i.e., germinal zones) of the E13.5–E14 MGE and LGE were separately dissected as previously described (Olsson et al., 1995). The VM was dissected from E12 mouse embryos as described by Nikkhah et al. (1994). Single-cell suspensions were prepared in 0.05% DNase/DMEM using either light trypsinization (0.1% trypsin for 15–20 min at 37°C) or, in most cases, mechanical dissociation. Timed pregnant Sprague–Dawley rats (BK Universal, Stockholm, Sweden) with embryos at a gestational age of E15–E15.5 were anesthetized with either Equithesin (3.0 ml/kg, intraperitoneally) or halothane (1.5% halothane/air mixture), and a midline laparotomy was performed. Maintaining sterility, only one uterine horn was removed from the abdominal cavity at a time, and each embryo was oriented in the uterine sac such that the telencephalic vesicles and calvarian sutures were evident by transilluminating the uterine sac (when the embryo was oriented at the correct angle to the light, the forebrain ventricles were clearly visible). Approximately 10^5 cells in 1 μl of either the LGE, MGE, or VM suspensions were deposited into the cerebral ventricle of the age-matched rat embryos (the E13.5–E14 mouse tissue is at a similar developmental stage as the E15–E15.5 rat embryos since the gestational period for mice is about 1–2 days shorter than that for rats). The injection was performed free hand (by one experimenter) using a 10

μl Hamilton syringe equipped with a glass micropipette (~60 μm outer diameter; Nikkhah et al., 1994). A second experimenter often assisted in holding the embryo in place. The injection was completed within seconds, and the needle was withdrawn following a delay of ~5–10 s. After transplantation, the uterine horns were placed back into the abdomen, and the mothers were sutured and left to give birth. Some of the mothers carrying embryos transplanted on E15 received injections of [^{3}H]thymidine (5 μCi/g; specific activity, 40 Ci/mmol; Amersham) 3 days later (at E18). Since M6, the marker used to trace the donor mouse cells, is present only on the surface membrane of postmitotic neural cells (Lund et al., 1985; Lagenauer et al., 1992; Yan et al., 1993), all animals were analyzed at postnatal stages. The percentage of embryos that survived into the postnatal period after transplantation in utero was ~70%. Of the surviving animals, ~40% possessed detectable amounts of mouse neural tissue in the recipient rat brain.

Immunohistochemical Detection of Grafted Cells

After birth, the injected rat pups were allowed to survive for 0–28 days before being sacrificed either by decapitation, followed by immersion-fixation of the brain in 4% paraformaldehyde (P0–P6 pups), or by transcardial perfusion with 4% paraformaldehyde (P7–P28). After fixation, the brains were placed into 30% (P0–P14) or 20% (P21–P28) sucrose in 0.1 M phosphate buffer before sectioning. The P0–P7 brains were cut at 20 μm thickness on a cryostat, while the older brains were sectioned at 30–40 μm thickness using a sliding freezing microtome. Donor mouse cells were identified using immunohistochemistry for the mouse neural marker M6 (Lund et al., 1985; Lagenaur et al., 1992) and the mouse astrocytic marker M2 (Lund et al., 1989; Zhou et al., 1990). In brief, after a 10 min incubation in 3% H_2O_2 and a subsequent 1 hr preincubation in 10% normal rabbit serum, the sections were incubated in rat monoclonal antibodies to M6 or M2 (courtesy of Dr. C. Lagenauer) at 1:50 overnight in 10% normal serum. The immunohistochemical product was detected by the ABC method using a biotinylated rabbit anti-rat antibody as described by the manufacturers (Vector labs), and the immunohistochemical product was visualized using 3′,3′-diaminobenzidine (DAB) as a chromogen. Since the secondary antibodies were directed against rat antigens, control stainings of adjacent sections were performed with the primary antibody (either M6 or M2) excluded from the staining protocol. In animals injected with [^{3}H]thymidine on E18 and stained for M6, the sections were dipped into liquid emulsion (Ilford K5; diluted 1:1) and exposed for 6–8 weeks before being developed in Kodak D19 at 18°C for 3 min and fixed for 10 min in AGFA G333 (AGFA-Gefvaert).

Double Immunohistochemistry

In the double staining procedure used to determine the neurochemical phenotypes of incorporated mouse neurons, the M6 staining was performed as above, except that the DAB reaction product was intensified using nickel to give a black product. After washing extensively, the sections were again bleached in H_2O_2 (to inactivate the peroxidase complex from the prior M6 staining) and incubated in either mouse anti-DARPP-32 (1:20000; a gift of Dr. P. Greengard), rabbit anti-CaBP (1:500), rabbit anti-parvalbumin (1:1000; generously provided by Dr. P. C. Emson), or mouse anti-p75 (which detects the low affinity nerve growth factor receptor; diluted 1:250; gift of Dr. E. Johnson). The ABC method (Vector Labs) was again used with the appropriate biotinylated secondary antibodies and DAB as the final chromogen. In the sections double stained with M6 and CaBP or parvalbumin (both raised in rabbits), the M6 incubation was performed in 10% normal horse serum (Lund et al., 1985; Zhou et al., 1990) since the normal rabbit serum produced a nonspecific high background when the anti-rabbit IgGs were used to detect calbindin or parvalbumin. All double staining was performed on sections adjacent to those singly stained for M6, and in all cases the pattern of M6 staining was identical to that on the adjacent sections. The labeling patterns for the neurochemical markers (DARPP-32, CaBP, parvalbumin, and p75) matched those reported previously by numerous investigators.

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Note Added in Proof

The data referred to as Götz et al., 1994, Soc. Neurosci., abstract, are now in press: Götz, M., Wizenmann, A., Reinhardt, S., Lumsden, A., and Price, J. (1996). Neuron, in press.